Remarks

Claims 50-56, 58-60, 62, 63, and 87-89 are pending in the subject application. Applicants acknowledge that claims 53, 62, 63, 88, and 89 have been withdrawn from further consideration as being drawn to a non-elected invention. By this Amendment, Applicants have canceled claim 51. Support for the amendment can be found throughout the subject specification and in the claims as originally filed. Entry and consideration of the amendments presented herein is respectfully requested. Accordingly, claims 50, 52-56, 58-60, 62, 63, and 87-89 are currently before the Examiner. Favorable consideration of the pending claims is respectfully requested.

Applicants gratefully acknowledge the Examiner's withdrawal of the rejections under 35 U.S.C. § 112, second paragraph. With respect to the issue raised regarding the substantially duplicative claims set forth in the Office Action, Applicants have canceled claim 51.

Claims 50-52, 54-56, 58-60, and 87 are rejected under 35 U.S.C. § 101 as lacking a specific asserted utility or a well established utility. The claims are also rejected under 35 U.S.C. § 112, first paragraph because the specification, allegedly, fails to teach how to use the claimed invention. The Office Action indicates that "the disclosed results of experiments fail to establish support of an assertion that a compound, which modulates KCNQ, has 'a pharmacological activity' with respect to any disease or disorder. As such, there appears to be no immediate need for a method to screen for compounds that modulate KCNQ activity." The Office Action also argues that the characterization of the interaction between KCNQ and PP2Q, as the polypeptides being expressed in brain and regulated by a drug known to treat bipolar disorder, is clearly not sufficient to establish the biological significance of this interaction to bipolar disorder or to any mental disorder in general. The Office Action also argues that the instant specification fails to present any evidence or sound scientific reasoning that the KCNQ/PP2A activity/binding has any specific physiological effect in the etiology or course of a mental disorder; thus, it is not reasonable to believe that the administration of a compound that modulates the activity or binding of KCNQ/PP2A would have any effect on the course of the disease. Applicants respectfully submit that the as-filed application contains a specific and credible asserted utility for the claimed polypeptide and/or a well established utility and traverse the rejections of record.

The Examiner bears the initial burden of showing that a claimed invention lacks patentable utility. *See In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995). ("Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility."). In this case, Applicants respectfully submit that the Patent Office has not met its burden of establishing why one of ordinary skill in the art would reasonably doubt the asserted utility. Rather, the Office Action indicates that "the disclosed results of experiments fail to establish support of an assertion that a compound, which modulates KCNQ, has 'a pharmacological activity' with respect to any disease or disorder. As such, there appears to be no immediate need for a method to screen for compounds that modulate KCNQ activity." The Office Action also argues that the characterization of the interaction between KCNQ and PP2Q, as the polypeptides being expressed in brain and regulated by a drug known to treat bipolar disorder, is clearly not sufficient to establish the biological significance of this interaction to bipolar disorder or to any mental disorder in general. Applicants respectfully submit that the conclusory statements made within the first Office Action and the final rejection issued in this matter fail to establish that one of ordinary skill in the art would reasonably doubt the asserted utility of the claimed invention and that the Patent Office has failed to establish that the claimed invention lacks patentable utility. Accordingly, reconsideration and withdrawal of the rejections of record is respectfully requested.

With respect to the assertion in the Office Action that the as-filed specification fails to disclose a role for the claimed polypeptide or its significance to a particular disease, disorder or physiological process that one would wish to manipulate for a desired effect, Applicants again reiterate that the as-filed specification indicates that the claimed polypeptides are candidate targets useful for the identification of candidate compounds suitable for the treatment of mental disorders such as bipolar disorder, schizophrenia, depression and other mood disorders (see page 1, paragraph 1 of the as-filed specification). Further, the specification also teaches (see page 14, paragraphs 1-2):

> The present invention stems from the cloning and the sequencing of three novel splice variants of the KCNQ2 gene, KCNQ2-15bx, KCNQ2-15by and KCNQ2-15bz. These splice variants all display a novel exon (exon 15b), corresponding to amino acids 545 to 643 of SEQ ID NO: 2. Data showing that

KCNQ2-15bx and KCNQ2-15by can assemble as functional homotetrameric potassium channels are provided. In the frame of the present invention, it has been demonstrated that these novel splice variants interact with the Bγ subunit of the serine/threonine protein phosphatase 2A (PP2A/Bγ) both *in vitro* and *in vivo*. Furthermore, association studies are described in example 15, and it was shown that both the KCNQ2 gene and the gene coding for PP2A/Bγ are strongly associated with bipolar disorder. Novel validated biallelic markers located in the KCNQ2 gene and associated with bipolar disorder are provided. In the frame of the present invention it was further shown that KCNQ2-15bx, KCNQ2-15by and KCNQ2-15bz are (i) dephosphorylated by PP2A; and (ii) phosphorylated by the PKA and GSK3β kinases. Moreover, the phosphorylation of KCNQ2-15bx, KCNQ2-15by and KCNQ2-15bz is inhibited in the presence of lithium, a known mood-stabilizing agent.

Accordingly, the present invention provides novel KCNQ2 polypeptides and means to identify compounds useful in the treatment of mental disorders such as bipolar disorder, schizophrenia, depression and other mood disorders. The invention further relates to the use of KCNQ2 polypeptides as targets for screening for modulators thereof. The use of said modulators for treating mental disorders, and the use of biallelic markers located in the KCNQ2 gene for diagnosing mental disorders are further aspects of the present invention.

While Applicants are of the position that the Patent Office has failed to establish that the claimed invention lacks patentable utility, it is respectfully submitted that Borsotto *et al.* (*The Pharmacogenomics J.*, 2007, 7:123-132, Abstract) provides a further discussion of KCNQ polypeptides disclosed within this application:

Many bipolar affective disorder (BD) susceptibility loci have been identified but the molecular mechanisms responsible for the disease remain to be elucidated. In the locus 4p16, several candidate genes were identified but none of them was definitively shown to be associated with BD. In this region, the PPP2R2C gene encodes the Bγ-regulatory subunit of the protein phosphatase 2A (PP2A-Bγ). First, we identified, in two different populations, single nucleotide polymorphisms and risk haplotypes for this gene that are associated to BD. Then, we used the Bγ subunit as bait to screen a human brain cDNA library with the yeast two-hybrid technique. This led us to two new splice variants of KCNQ2 channels and to the KCNQ2 channel itself. This unusual K^+ channel has particularly interesting functional properties and belongs to a channel family that is already known to be implicated in several other monogenic diseases. In one of the BD populations, we also found a genetic association between the KCNQ2 gene and BD. We show that KCNQ2 splice variants differ from native channels by their shortened C-terminal sequences and are unique as they are active and exert a dominant-negative effect on KCNQ2 wild-type (wt)

> channel activity. We also show that the PP2A-Bγ subunit significantly increases the current generated by KCNQ2wt, a channel normally inhibited by phosphorylation. The kinase glycogen synthase kinase 3 beta (GSK3β) is considered as an interesting target of lithium, the classical drug used in BD. GSK3b phosphorylates the KCNQ2 channel and this phosphorylation is decreased by Li^+.

Borsotto *et al.* further indicate that their results suggest that a dysfunction of the KCNQ2-PP2A-Bγ subunit regulation pathway and/or a dysfunction in the KCNQ2/KCNQ2 splice variant expression and association might be responsible for changes in neuronal excitability that underline the manic and the depressive episodes occurring in BD (see page 130, column 1, paragraph 1, first sentence). Borsotto *et al.* also indicate that Li^+ has no effect on splice variant activity which could be related to the lack of efficacy of the lithium treatment in a significant number of bipolar disorder patients (see paragraph bridging pages 129-130). Thus, it is respectfully submitted that Borsotto *et al.* support the asserted utility that the polypeptide of SEQ ID NO: 2 as a candidate target protein suitable for use in screening assays to identify candidate compounds suitable for the treatment of bipolar disorder. Accordingly, reconsideration and withdrawal of the rejections of record is respectfully requested.

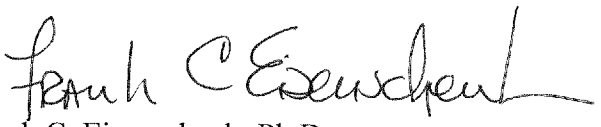
It should be understood that the amendments presented herein have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of Applicants' agreement with or acquiescence in the Examiner's position. Applicants expressly reserve the right to pursue the invention(s) disclosed in the subject application, including any subject matter canceled or not pursued during prosecution of the subject application, in a related application.

In view of the foregoing remarks and amendments to the claims, Applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



Frank C. Eisenschenk, Ph.D.
Patent Attorney
Registration No. 45,332
Phone No.: 352-375-8100
Fax No.: 352-372-5800
Address: P.O. Box 142950
Gainesville, FL 32614-2950

FCE/sl
Attachment: Copy of Borsotto *et al.*, 2007

The Pharmacogenomics Journal (2007) 7, 123–132

www.nature.com/tpj

ORIGINAL ARTICLE

PP2A-Bγ subunit and KCNQ2 K^+ channels in bipolar disorder

M Borsotto[1,3], L Cavarec[2,3], M Bouillot[2], G Romey[1], F Macciardi[2], A Delaye[2], M Nasroune[2], M Bastucci[2], J-L Sambucy[2], J-J Luan[2], A Charpagne[2], V Jouët[2], R Léger[2], M Lazdunski[1], D Cohen[2] and I Chumakov[2]

[1]*Institut de Pharmacologie Moléculaire et Cellulaire, CNRS-Université de Nice, Institut Paul Hamel, Sophia Antipolis, Valbonne, France and* [2]*Serono Genetics Institute SA, 5 rue Henri Desbruères, Evry cedex, France*

Correspondence:
Professor M Lazdunski, Institut de Pharmacologie Moleculaire et Cellulaire, IPMC CNRS UMR 6097, 660 Route des Lucioles, Sophia Antipolis, Valbonn 06560, France.
E-mail: lazdunski@ipmc.cnrs.fr

[3]These authors contributed equally to this work.

Received 10 January 2006; revised 22 March 2006; accepted 18 April 2006; published online 30 May 2006

Many bipolar affective disorder (BD) susceptibility loci have been identified but the molecular mechanisms responsible for the disease remain to be elucidated. In the locus 4p16, several candidate genes were identified but none of them was definitively shown to be associated with BD. In this region, the *PPP2R2C* gene encodes the Bγ-regulatory subunit of the protein phosphatase 2A (PP2A-Bγ). First, we identified, in two different populations, single nucleotide polymorphisms and risk haplotypes for this gene that are associated to BD. Then, we used the Bγ subunit as bait to screen a human brain cDNA library with the yeast two-hybrid technique. This led us to two new splice variants of KCNQ2 channels and to the KCNQ2 channel itself. This unusual K^+ channel has particularly interesting functional properties and belongs to a channel family that is already known to be implicated in several other monogenic diseases. In one of the BD populations, we also found a genetic association between the *KCNQ2* gene and BD. We show that KCNQ2 splice variants differ from native channels by their shortened C-terminal sequences and are unique as they are active and exert a dominant-negative effect on KCNQ2 wild-type (wt) channel activity. We also show that the PP2A-Bγ subunit significantly increases the current generated by KCNQ2wt, a channel normally inhibited by phosphorylation. The kinase glycogen synthase kinase 3 beta (GSK3β) is considered as an interesting target of lithium, the classical drug used in BD. GSK3β phosphorylates the KCNQ2 channel and this phosphorylation is decreased by Li^+.
The Pharmacogenomics Journal (2007) **7**, 123–132. doi:10.1038/sj.tpj.6500400; published online 30 May 2006

Keywords: PP2A-Bγ; KCNQ2; lithium; GSK3β; bipolar disorder

Introduction

Bipolar affective disorder (BD) is a common psychiatric disease which afflicts 0.5–1.5% of the world population irrespective of the culture and geographical origin.[1] It is mainly characterized by episodes of mania and/or hypomania interspersed with periods of depression. Heritability of BD is well documented and a number of susceptibility loci have been identified on several chromosomes but, up to date, no specific genes were found. Among these regions we retained the 4p16 locus since significant linkage with BD was found in Scottish, Danish and American pedigrees.[2–5] A recent study on Faroese population confirmed the link between this locus and BD and schizophrenia.[6] The 4p16 region encodes several interesting candidate genes such as *ADRA2C* (α-adrenergic receptor 2C), *WFS1* (wolframin; heterozygous carriers display an estimated 26-fold increased incidence of psychiatric illness[7]) or *DRD5* (dopamine D5 receptor) genes.

Nevertheless, none of them has been definitively proven to participate in BD.[8–11] Thus, we tested a region around the *WFS1* gene for genetic association with BD by case/control single nucleotide polymorphism (SNP) markers association study. We found that SNPs in the *PPP2R2C* gene are associated with BD. Interestingly the microsatellite marker D4S431 located within this gene is the telomeric mark of a D4S431–D4S403 region where Blackwood *et al.*[2] originally observed the occurrence of the maximum lod score in a Scottish pedigree.

The *PPP2R2C* gene encodes the Bγ regulatory subunit of protein phosphatase 2A (PP2A-Bγ). The expression pattern of this subunit is brain and neuron specific,[12] where, as other regulatory subunits, it acts by targeting specific substrates to the PP2A core enzyme. This enzyme is associated with phosphorylation–dephosphorylation processes which, when they do not operate properly, might easily lead to neuronal diseases.[13,14] It was thus important to identify proteins associated with PP2A-Bγ, and eventually link the genetic data to a functional hypothesis. This type of strategy has previously been used to identify candidate genes and their partners in schizophrenia.[15] Among the different partners identified to interact with PP2A-Bγ, the present study identifies and describes the KCNQ2 potassium channel and two new splice variants. The interest for this particular partner of PP2A-Bγ was guided by the following reasons (i) KCNQ2 channel activity is dependent on its state of phosphorylation,[16] (ii) dysfunction of ionic channels associated with pathologies[17] has been observed for several members of the KCNQ family, particularly mutations in KCNQ2 channels are responsible for a peculiar form of epilepsy called benign familial neonatal convulsions (BFNC),[18,19] (iii) a genetic association study indicates that *KCNQ2* gene is also linked to BD in one of the two populations we used.

Results

Genetic analysis for PP2A-Bγ subunit

Four SNP markers have been developed in the *PPP2R2C* gene and genotyped in two independent collections of BD patients from Argentina and UK. We have shown that these markers follow the Hardy–Weinberg conditions of gametic equilibrium. Allelic and genotypic *P*-values were calculated for these markers with traditional χ^2 statistics and we considered results with *P*-values $<5\times10^{-2}$ as nominally significant. We found that all four SNPs are associated with BD (Table 1, significant data in bold). SNP 99-24169/139 is associated with BD in the UCL collection (significant allelic and genotypic *P*-values), while SNPs 24-257/320 and 99-24175/218 are associated in the Labimo collection (significant allelic *P*-values). In addition, 99-24175/218 and 24-247/216 are also associated with BD in the UCL collection with a significant genotypic *P*-value (Table 1).

In order to determine if there is a combination of SNPs that presents a higher risk of developing the disease, we performed a haplotype analysis for 24-257/320 and 99-24175/218 in the Labimo collection and for 99-24169/139 and 24-247/216 in the UCL collection. Results (Supplementary information, Table 3) indicate that the risk haplotype for 24-257/320 and 99-24175/218 is "AA" and that for 99-24169/139 and 24-247/216 is "AG". Thus, an individual carrying the haplotype 'AA' at biallelic markers 24-257/320 and 99-24175/218 or the haplotype 'AG' at biallelic markers 99-24169/139 and 24-247/216 presents an increased risk of developing BD. These results suggest that extended predisposition alleles linked to BD disease through linkage disequilibrium could code for functionally different (mutated) alleles of this gene that contribute to the pathogenesis of BD.

Identification of novel isoforms of the KCNQ2 voltage-gated potassium channel by yeast two-hybrid screening

With the aim of identifying new protein targets of the PP2A-Bγ subunit with a functional link to BD, we screened an adult human brain cDNA library with the yeast two-hybrid (Y2H) system using a bait constructed with the full-length cDNA of the *PPP2R2C* gene. We screened 1.2×10^7 yeast transformants of the cDNA library. We analyzed a total of 494 DNA sequences among which 122 code for 43 independent genes (Supplementary information, Table 7). Seven identified genes coded for cytoskeletal or related proteins such as MAP1A LC2, MAP1B LC1, tubulin β4, β-actin, SCG10/stathmin 2, flotillin-1 or dynein light chain A. The nature and functions of these putative partners corresponded to the cytoskeletal localization of PP2A-Bγ subunit and consequently, validate the reliability of the Y2H

Table 1 *P*-values for biallelic markers located in PPP2R2C

Biallelic marker name	*Location in PPP2R2C*	*Collection*	*Chosen allele*	*Allelic frequency difference*	*Allelic odds ratio*	*Allelic P-value*	*Genotypic P-value*
99-24169/139	Intron 1d	UCL	A	0.095	1.733	**2.19×10^{-4}**	**3.61×10^{-4}**
		Labimo	A	0.002	1.012	9.46×10^{-1}	5.98×10^{-1}
24-247/216	Intron 4	UCL	G	0.047	1.275	7.75×10^{-2}	**2.29×10^{-2}**
		Labimo	G	0.024	1.125	4.86×10^{-1}	7.65×10^{-1}
24-257/320	Intron 5	UCL	A	0.018	1.079	5.52×10^{-2}	8.22×10^{-1}
		Labimo	A	0.102	1.557	**4.04×10^{-3}**	1.19×10^{-2}
99-24175/218	Intron 5	UCL	G	0.035	1.162	2.62×10^{-1}	**3.99×10^{-3}**
		Labimo	A	0.096	1.546	**6.69×10^{-3}**	2.34×10^{-2}

Significant values are in bold

screen. Three other clones coding for proteins involved in the control of the cellular electrical activity have been identified. They correspond to the β1 subunit of the Na^+/K^+ adenosine triphosphate (ATP)ase, the synaptosomal-associated protein 25 and the KCNQ2 K^+ channel. We focused our attention on two independent clones (3H2 and 3H9) which were of particular interest because they both correspond to partial polyadenylated cDNAs encoding new splice variants of the intracytoplasmic domain of the voltage-gated potassium channel KCNQ2.

An alignment of the longest clone 3H2 with the genomic sequence of KCNQ2, showed that it corresponds to full-length exons 12–14, and to the unspliced intron 14 according to the nomenclature of Biervert and Steinlein.[20] Despite the lack of intron 14 splicing-out, this latter remains in frame with exon 14, over 55 amino-acid residues. The new encoded intracytoplasmic domain, although shorter than that of the canonical KCNQ2 isoform 1 (KCNQ2 wild type (wt)),[19] presents a complete A-domain and a large part of the B-domain described as essential for channel functionality[21] (Figure 1a and Supplementary information, Figure 1a). Real-time polymerase chain reaction (PCR) performed on human brain cDNAs indicates that splice variant mRNAs represent about 10% of wt channel mRNAs (Supplementary information, Figure 1b).

Mating experiments were duplicated and, using the same bait, we again isolated 3H2 and 3H9 clones, confirming the relevance of the interaction detected during the initial Y2H screen.

Genetic analysis for KCNQ2

In order to strengthen the relevance of the implication of the KCNQ2 channel in BD, we calculated the genetic association of the *KCNQ2* gene with bipolar disease by case/control SNP markers association study. Six SNPs located in the *KCNQ2* gene were found from Celera data bank and genotyped in the two independent collections of BD previously used for the *PPP2R2C* genetic study. No association was found in the Argentinian Labimo collection whatever the SNP tested. Nonetheless, two SNPs, 30-2/62 and 30-7/30, within the *KCNQ2* gene are significantly associated with BD in the UCL collection (Table 2). More specifically, 30-2/62 was found to be highly associated with BD in this collection, with both significant allelic and genotypic *P*-values (Table 2). The risk genotype for 30-2/62 is 'AG' and that for 30-7/30 is 'CC'. The significant genotypic odds ratios for 30-2/62 are 1.62 (AG versus (GG + AA)) and 1.82 ((AG + AA) versus GG). For the 30-7/30 marker they are of 1.58 and 1.71 for (CC versus TT) and (CC versus (TT + CT)) respectively (Supplementary information, Table 6). The association results of the single biallelic marker frequency analysis show that the *KCNQ2* gene is associated with BD. These results validate the hypothesis of a molecular interaction between PP2A-Bγ -regulatory subunit and KCNQ2 channels, consequently, a dysfunction and/or a dysregulation of these channels, associated or not with PP2A-Bγ dysfunction, may contribute to the onset and development of BD.

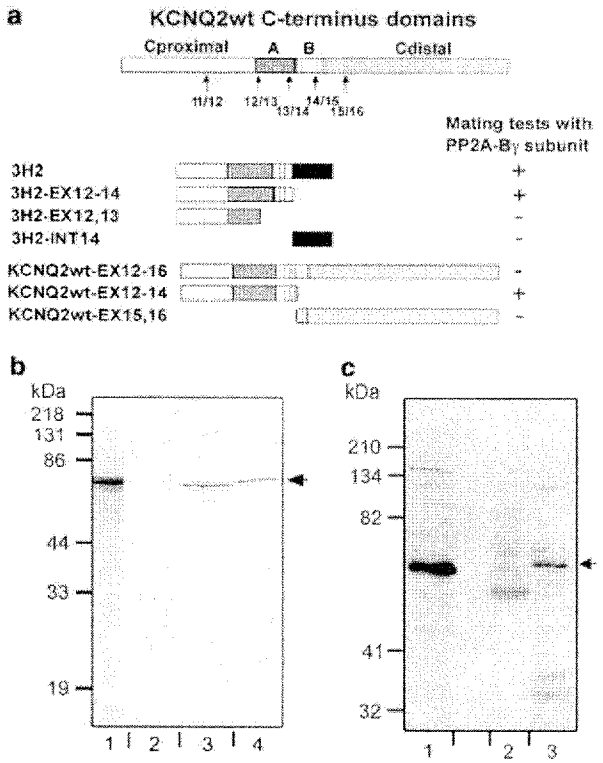


Figure 1 C-terminus sequence organization and GST pull-down assays. (**a**) A schematic organization of the KCNQ2wt C-terminus domains and fragments fused to a GAL4-activating domain and tested for interaction with the PP2A-Bγ regulatory subunit. Activation of histidine growth reporter is indicated by + and −. A domain (dark gray box) and B or B truncated domains (streaked boxes) are visualized. The translated product of intron 14 is represented by a black box. (**b**) GST pull-down assay. *In vitro* translated and [^{35}S]-labelled full-length PP2A Bγ -regulatory subunit (lane 1, crude translated product) was incubated with either GST (lane 2) or GST-3H2 (lane 3) or GST-KCNQ2wt-Cter (lane 4) immobilized on glutathione beads. Labelled proteins were revealed by autoradiography. PP2A-Bγ subunit is indicated by an arrow. (**c**) Association of the 3H2 product and the PP2A-Bγ -regulatory subunit in HEK-293 cells. Lane 1, total cell extract. GST alone (lane 2) and GST-3H2 (lane 3) were coupled to glutathione-Sepharose beads and incubated with extract from cells stably overexpressing HA-tagged PP2A Bγ -regulatory subunit (arrow), which was detected with an anti-HA monoclonal antibody.

Interaction domain mapping of the 3H2 and KCNQ2wt products.

Using mating test experiments we have characterized the domains necessary for the interaction between KCNQ2 channels that is wt and clone 3H2, and the PP2A-Bγ subunit (Figure 1a). We found that the 55 amino acids encoded by the unspliced intron 14 of KCNQ2 were not necessary for the interaction between the 3H2 product and the PP2A-Bγ subunit. Only the 158 amino residues encoded by exons 12–14 are responsible for the association. Amino acids of exon 14, spanning the junction between the A- and B-domains, are crucial for binding as their removal totally abolished the occurrence of colonies on the most stringent selective medium.

Table 2 *P*-values for biallelic markers located in the KCNQ2 gene

Marker name	*Location in the KCNQ2 gene*	*Collection*	*Chosen allele*	*Allelic frequency difference*	*Allelic odds ratio*	*Allelic P-value*	*Genotypic P-value*
4/30/1958	5′ end	UCL	—	—	—	—	—
		Labimo	G	0.03	1.24	3.01×10^{-1}	5.85×10^{-1}
30-2/62	Intron 1	UCL	A	0.05	1.23	$\mathbf{7.76 \times 10^{-2}}$	$\mathbf{5.20 \times 10^{-3}}$
		Labimo	A	0.03	1.13	4.42×10^{-1}	1.15×10^{-1}
30-17/37	Intron 4	UCL	A	0.01	1.03	7.77×10^{-1}	9.12×10^{-1}
		Labimo	G	0.03	1.13	4.70×10^{-1}	7.10×10^{-1}
7/30/1930	Intron 12	UCL	C	0.05	1.21	1.05×10^{-1}	$\mathbf{3.02 \times 10^{-2}}$
		Labimo	C	0.02	1.06	7.03×10^{-1}	5.32×10^{-1}
30-84/37	3′ end	UCL	A	0.02	1.20	3.06×10^{-1}	3.69×10^{-1}
		Labimo	—	—	—	—	—
30-15/54	3′ end	UCL	A	0.01	1.06	6.92×10^{-1}	7.68×10^{-1}
		Labimo	—	—	—	—	—

Significant values are in bold

Using similar assays we were unable to show interaction between the Bγ subunit and products of exons 12–16 or exons 15 and 16 of KCNQ2wt whereas products of exons 12–14 were able to interact (Figure 1a).

In vitro and ex vivo protein–protein interaction assays: glutathione S-transferase pull-down and solid phase overlay

The interaction of the PP2A-Bγ subunit with the 3H2 product was confirmed by glutathione *S*-transferase (GST) pull-down experiments. [^{35}S]-Bγ subunit was precipitated by the GST-3H2 fusion protein but not by GST protein alone (Figure 1b). This association was confirmed by a solid phase overlay assay indicating that the interaction is direct (Supplementary information, Figure 3). Interestingly, a GST fusion protein prepared with the C-terminal part of KCNQ2wt was also able to precipitate the [^{35}S]-Bγ subunit (Figure 1b).

The association of the PP2A-Bγ regulatory subunit with the product of the 3H2 clone was further tested in stably transfected HEK-293 cells overexpressing pHM6-PPP2R2C (HEK-293-PP2A-Bγ), coding for a N-terminal HA-tagged version of PP2A-Bγ subunit. The GST-3H2 peptide precipitated a protein that was detected by anti-HA antibodies and with a size corresponding to the tagged version of PP2A-Bγ subunit (Figure 1c). No PP2A-Bγ protein was precipitated by GST alone.

Both *in vitro* assays and *ex vivo* precipitation experiments confirmed the relevance of the interaction identified *in vivo* by the Y2H system.

Cloning of two full-length cDNAs corresponding to the new splice variant isoform of the voltage-gated potassium channel KCNQ2

Full-length cDNAs encoding the new KCNQ2 splice variants were isolated from reverse transcription-polymerase chain reactions performed with human brain thalamus polyA+ mRNA and specific primers of both 3H2 clone and KCNQ2wt. Two cDNAs, hereafter called Q2-3H2 and Q2-3H9, respectively 1933 and 1879 bp long, were entirely sequenced. Q2-3H9 cDNA differs from Q2-3H2 only by the absence of exon 11. Owing to the lack of intron 14 splicing-out the intracytoplasmic domains of both Q2-3H2 and Q2-3H9 are shorter (331 and 313 amino residues, respectively) than that of KCNQ2wt (560 amino residues) (Figure 1a and Supplementary information, Figure 1a). No clones corresponding to another unspliced intron were found among numerous clones we sequenced, arguing against cloning artifacts for both Q2-3H2 and Q2-3H9 splice variants.

Membrane expression of the new KCNQ2 splice variant isoforms

Q2-3H2 and Q2-3H9 splice variants are expressed at the cell surface as demonstrated by direct immunocytochemistry experiments performed on HEK-293 transiently transfected with V5-tagged channels showing that both permeabilized and non-permeabilized cells display a strong signal (Figure 2Aa,b and Supplementary information, Figures 2A and 4). The apparent decrease of cell surface expression in non-permeabilized cells is also observed with KCNQ2wt V5-tagged channels (Figure 2Ac,d).

Homo or heteromeric associations are a necessary property of the KCNQ family to be expressed at the cellular membrane.[21,22] These interactions take place in the C-terminal part of channels. We checked, using GST pull-down technique, that both variants were able to homomerize and heteromultimerize with wt channels (Supplementary information, Figures 2B and 5).

Taken together these experiments indicate that the new splice variant isoforms are correctly expressed at the cell membrane and are able to form heteromultimers with the KCNQ2wt channel, two necessary criteria for channel activity.

COS cell expression of KCNQ2wt and splice variant channels

The whole cell patch-clamp technique was used to record currents from transfected cells, the CD8 antigen was used as a transfection reporter. We found that 100% of CD8-positive cells expressed the KCNQ2wt channel, that only 10% of CD8-positive cells also expressed the Q2-3H2 channel and surprisingly, that 0% expressed the Q2-3H9 channel (Figure

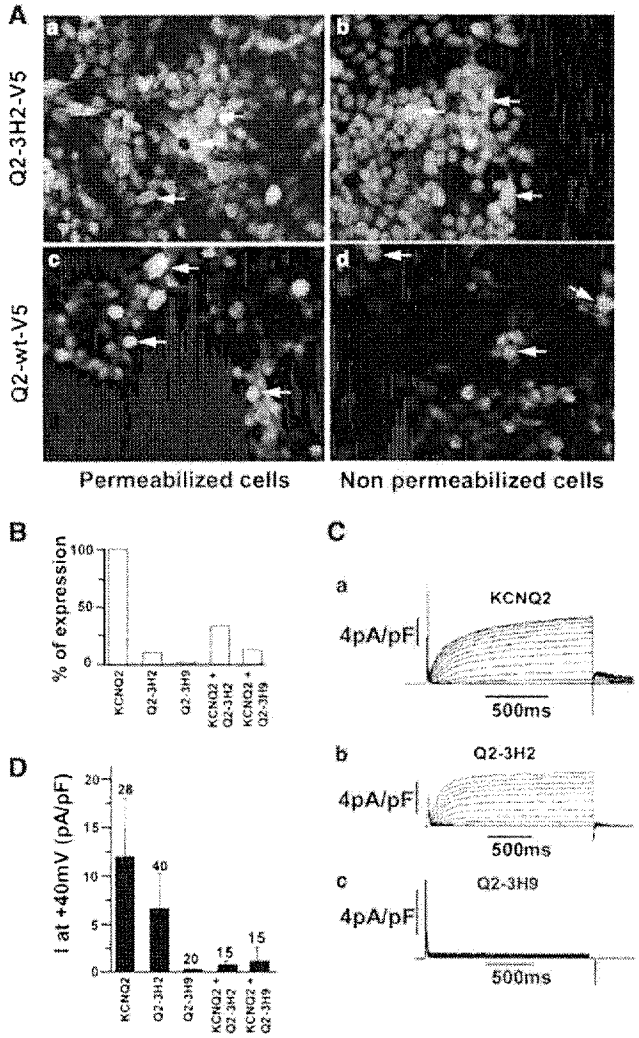


Figure 2 The Q2-3H2 variant is expressed at the cell surface. (**A**) Immunofluorescence analysis of HEK-293 cells. V5-tagged Q2-3H2 (a and b) or V5-tagged KCNQ2wt (c and d) were visualized with a monoclonal anti-V5 antibody coupled to an FITC-anti-V5 antibody (green) and DNA (red) was revealed using the DAPI method in permeabilized (a and c) or non-permeabilized (b and d) conditions. (**B**) Percent of cells displaying a current among CD8-positive cells. (**C**) Expression of KCNQ2wt and Q2-3H2 and Q2-3H9 splice variant channels in mammalian COS7 cells. (**D**) Dominant-negative effects of splice variants on KCNQ2wt channel expressed in COS7 cells. Current values were measured at +40 mV with KCNQ2wt, Q2-3H2, Q2-3H9, KCNQ2wt + Q2-3H2, KCNQ2wt + Q2-3H9; bars represent s.e.m. Representative traces of currents recorded with KCNQ2wt (a), Q2-3H2 (b) and Q2-3H9 (c) channels. Holding potential: −80 mV, $\Delta V = 10$ mV.

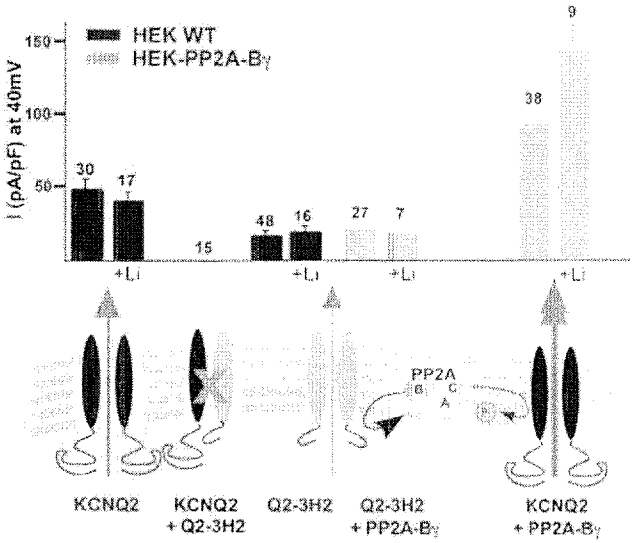


Figure 3 Schematic representation of the different channel complexes and their relative current densities when expressed in HEK wild-type (black boxes) and PP2A-Bγ (gray boxes) cells. Currents at +40 mV were recorded 48 h after transfection. The error bars indicate s.e.m., numbers at the top of the bar indicate the number of tested cells. The current produced by the homomeric Q2-3H2 channels is not affected by the presence of PP2A-Bγ subunit. +Li indicates that experiments were performed after 16 h of a 10 mM LiCl treatment. KCNQ2wt is represented in black and Q2-3H2, in gray.

2B). To check that these differences were not due to the cell line used, we repeated these experiments in the CHO cell line and in Xenopus oocytes and we obtained very similar results (data not shown).

3H2 mutant kinetics were faster than those of the wt channel (Figure 2C), 100–180 versus 250–700 ms and 50–120 versus 200–400 ms for activation and deactivation kinetics, respectively. Activation curves were not significatively different since the voltage to obtain 50% of activation ($V_{0.5}$) remains in the same range towards −20 mV (Supplementary information, Figure 6).

Cotransfection of KCNQ2wt with either Q2-3H2 or Q2-3H9 splice variants resulted in the absence of measurable currents (Figure 2D) indicating the dominant-negative effect of splice variants on wt channel activity. These data were confirmed in Xenopus oocyte experiments (data not shown).

The possible interaction of Q2-3H2 or Q2-3H9 with the KCNQ3 channel (the KCNQ2 M-type channel partner) was also tested in Xenopus oocytes. We found that both splice variants are without effect on KCNQ3 channel activity (Supplementary information, Figure 7).

Functional effects of PP2A-Bγ expression on the KCNQ2 channel activity

Since the interaction between PP2A-Bγ and KCNQ2 channels (wt and splice variants) was demonstrated *in vitro*, we decided to analyze the potential functional role of such an association. We transfected the K^+ channels in the PP2A-Bγ-HEK cells and compared results with those obtained after transfection in wt-HEK cells. Despite its high affinity observed in Y2H experiments, PP2A-Bγ had no effect on the Q2-3H2 splice variant activity. Current density values were 17 ± 4 and 15 ± 3 pA/pF in PP2A-Bγ- and wt-HEK cells, respectively (Figure 3). Conversely, PP2A-Bγ increased by a factor of about two the current generated by the KCNQ2wt.

Current density values were 88±9 and 45±5 pA/pF in PP2A-Bγ and wt-HEK cells, respectively (Figure 3). These functional results confirm the interaction observed in GST pull-down experiments.

A possible explanation of these results is that in HEK cells KCNQ2 channels are mostly present in a hyperphosphorylated/inactive state.[23] Thus, increasing the level of PP2A-Bγ subunit, and consequently phosphatase activity, would be expected to lead to an increase of the channel activity.

The glycogen synthase kinase 3 beta kinase phosphorylates the intracytoplasmic domain of KCNQ2

Because Li^+ is an important drug in the treatment of BD and because one of the possible Li^+ targets in mental disorders is the glycogen synthase kinase 3 beta (GSK3β) (Figure 4a),[24,25] we decided to check whether this particular kinase phosphorylates the channel. GSK3β is a peculiar serine/threonine kinase as it needs a prior phosphorylation by another protein kinase of another Ser or Thr residue at position +4 in order to be active.[26] Among kinases that were previously described to modulate the KCNQ2 activity,[16,27] we chose protein kinase A (PKA) as companion kinase. There are putative GSK3β phosphorylation sites (Supplementary information, Figure 1a) that are common to KCNQ2wt and to its splice variant discovered in this work. We chose the GST-3H2 construct to perform the phosphorylation assays. A band corresponding to the molecular weight of the GST-3H2 fusion protein was detected and only detected in the case of a synergistic phosphorylation by PKA and GSK3β (Figure 4b). GST alone was not radiolabelled (Figure 4b). Moreover, this phosphorylation was completely inhibited by addition of either a phospho-CREB-competitive substrate or by whole cell extracts prepared from the human glioblastoma/astrocytoma U-87 MG cell line which contain large amounts of phosphatase activity, including PP2A. This extract lost its efficacy in the presence of okadaic acid, a potent inhibitor of both protein phosphatases 1 and 2A (Figure 4b and Supplementary information, Figure 8). Lithium also partially inhibited GST-3H2 protein phosphorylation (Figure 4b and Supplementary information, Figure 9). Nevertheless, while lithium has no effect on KCNQ2wt expressed in HEKwt cells (Figure 3), we showed that, in PP2A-Bγ-HEK cells, the KCNQ2wt current (which is increased as compared with the corresponding current in wt cells, see above), is increased by about 50% after lithium treatment (current density values were 136±19 pA/pF, Figure 3). Lithium is without effect on Q2-3H2 channel activity in wt or PP2A-Bγ cells (Figure 3).

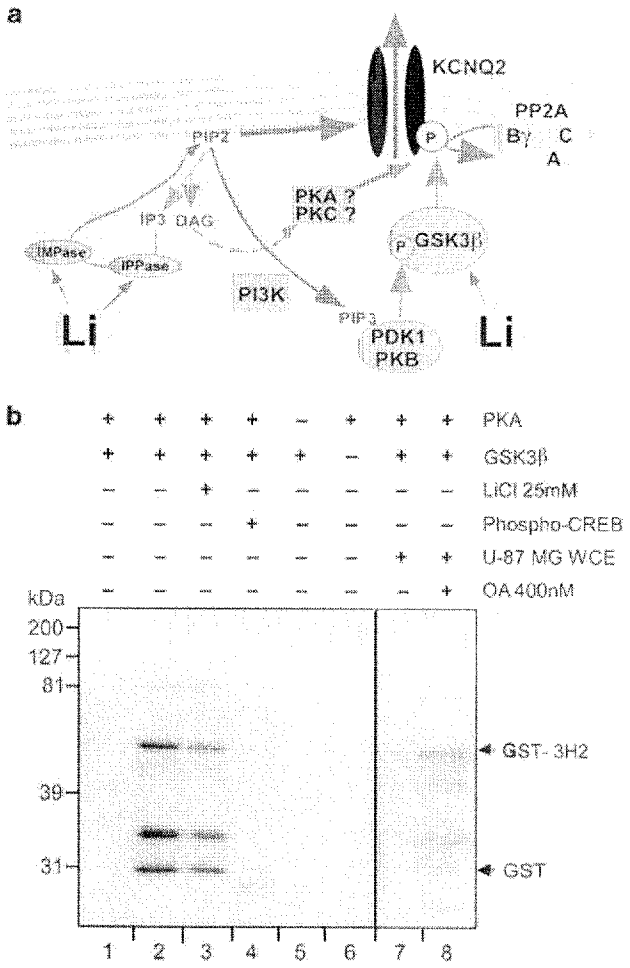


Figure 4 (**a**) Schematic representation depicting the lithium targets that could be implicated in the KCNQ2-regulating pathway. On one hand, Li^+ inhibits enzymes involved in the phosphoinositide biphosphate (PIP2) synthesis such as inositol monophosphate phosphatase (IMPase) or inositol polyphosphate and -phosphatase (IPPase). On the other hand, Li^+ also inhibits the GSK3β kinase that we show (see **b** below) is capable to phosphorylate the KCNQ2 channel. PI3K, phosphatidylinositol 3 kinase; PDK1, protein dependent kinase 1; PKA, PKB and PKC, protein kinase A, B and C; IP3, inositol 3 phosphate, DAG, diacyl glycerol. (**b**) Effects of GSK3β, PP2A-Bγ activities and lithium treatment on KCNQ2wt and spliced channels. The intracytoplasmic domain of KCNQ2 variants is phosphorylated *in vitro* by the GSK3β and dephosphorylated by PP2A. Synergistic *in vitro* phosphorylation by GSK3β and PKA of the GST-3H2 fusion protein. GST (lane 1) and GST-3H2 (lanes 2–6) proteins purified on glutathione-Sepharose 4B beads were first phosphorylated by protein kinase A (PKA) with non-radioactive ATP, and then by GSK3β with $^{32}P\gamma ATP$ (lanes 1–5). This second kination step was performed with 25 mM LiCl (lane 3) or 2.5 μg of the CREB phosphopeptide (lane 4) as control for the specific activity of GSK3β. Dephosphorylation of synergistically phosphorylated GST-3H2 fusion protein occurs when experiments are performed in the presence of a whole-cell extract prepared from the human glioblastoma/astrocytoma U-87 MG cell line (lane 7), these effects are inhibited by 400 nM okadaic acid, a potent inhibitor of PP1A and PP2A enzymes (lane 8). Positions of the radiolabelled GST-3H2 fusion protein and GSTalone are indicated by arrows.

Discussion

Among the loci that are genetically linked to BD, the chromosomal 4p16 region is found in several extended pedigrees (our data,[5,28]). We focused on the *PPP2R2C* gene that encodes the brain-specific PP2A-Bγ regulatory subunit.[12,29] Using case/control genetic association study in two different populations of bipolar patients, we found that individual SNPs and risk haplotypes in *PPP2R2C* gene are

associated to BD. The calculated odds ratios (OR, $1.546 < OR < 1.733$) are compatible with a multifactorial and multigenic disease which is not inherited following the classical Mendelian rules. OR values recently found for a candidate protein associated with insulin resistance are of 1.56 for type II diabetes and ranged between 1.37 and 1.69 for childhood or adult obesity.[30]

To understand how the *PPP2R2C* gene might be implicated in the pathology, we searched for interacting proteins by performing a Y2H screening of a human brain cDNA library with the PP2A-Bγ subunit as a bait. Among positive clones, we focused our attention on two new splice variants of brain-specific KCNQ2 channels,[18,31] Q2-3H2 and Q2-3H9. As these splice variants were both identified in two separate cDNA libraries, a cloning artifact can be excluded. No precise localization of the PP2A-Bγ subunit in human brain is available, however, the rat and mouse brain PP2A-Bγ subunit is found expressed in the same areas as the KCNQ2 channel.[12,32–34] The choice of KCNQ2, among other potentially interacting proteins, was motivated in part by the fact that the KCNQ channel family is of particular importance in human K^+ channelopathies. Among the 10 K^+ channel genes that have been associated with human genetic diseases, four belong to the KCNQ subfamily.[35,36] Mutations in KCNQ2, but also in KCNQ3 that can form heteromeric association with KCNQ2, are responsible for the same epileptic disorder, BFNC.[37,38] Owing to their slow deactivation kinetics, M-type channels act as 'damping' components that prevent neuronal hyperactivity. A reduction by only 25% of KCNQ2/KCNQ3 currents is enough to reach an epileptogenic level.[27] Interestingly, a susceptibility locus for BD was recently identified on chromosome 8q24, a region that contains the *KCNQ3* gene.[39] We tested the effects of Q2-3H2 or Q2-3H9 on KCNQ3 channel activity and we found that currents generated by the channel are unchanged in the presence of either of the splice variants (Supplementary information, Figure 7). These data indicate that these proteins probably do not interact. This observation is not surprising since the C-terminal domain of the channels are responsible for the KCNQ2/KCNQ3 heteromerization in M-type channels and this domain is not only shortened, but also different in the splice variants. Nevertheless, the identification of the KCNQ3 gene as a susceptibility locus for BD adds weight to the idea that the M-type channel (KCNQ2/KCNQ3) and its regulation might be involved in this disease.

The direct interaction between PP2A-Bγ and KCNQ2 variants was definitively established by GST pull-down and solid-phase overlay experiments. However, KCNQ2wt also associates with PP2A-Bγ as demonstrated by GST pull-down experiments. This interaction may be less tight that the interaction with KCNQ2 variants as it was not detected in the Y2H screening. Moreover, we performed genetic association study for the *KCNQ2* gene itself. Results show, at least in the UCL collection previously used for the *PPP2R2C* study, that two SNPs in the *KCNQ2* gene are associated to BD.

When expressed in mammalian cells, Q2-3H2 produces a current that resembles the wt current; however, activation and deactivation kinetics are three- to fivefold faster for the splice variants. Both Q2-3H2 and Q2-3H9 act as dominant-negative subunits for the expression of KCNQ2wt. Q2-3H2 and Q2-3H9 are unique among the number of described KCNQ2 splice variants,[31,40] in generating a K^+ current, but also exerting a dominant-negative effect. Splice variants identified in this work, because of their faster deactivation kinetics can only control the shape and duration of single action potentials. They cannot prevent the occurrence of fast bursts of action potentials. These properties associated with their dominant-negative effects on the wt KCNQ2 should be expected to confer a hyperexcitability to neurons where they are expressed. An increased excitability of neurons is a characteristic of manic and/or hypomanic periods of BD.[41] A recent report[42] has shown that the suppression of M channels with a KCNQ2 dominant-negative form not only results in a hyperexcitability of neurons and morphological changes of some brain structures, mainly hippocampus, but interestingly, also a decline of cognitive processes and behavioral changes corresponding to hyperactivity.[42]

KCNQ2 expression is dependent on phosphorylation state. When phosphorylated, the channel becomes inactive, while dephosphorylation increases its activity. Different kinases such as PKA[27] or PKC[16] are involved in the phosphorylation process, but little is known about the mechanism of dephosphorylation. Electrophysiological experiments show that, in HEK cells, PP2A-Bγ increases the KCNQ2wt current amplitude (Figure 3). Since in HEK cells, KCNQ2 preferentially exists in a phosphorylated/inactive state,[23] these results suggest that PP2A, via its Bγ-regulatory subunit stimulates the dephosphorylation of KCNQ2wt, and then increases channel activity. This current increase is not observed with the Q2-3H2-spliced isoform suggesting, in this case, a regulation pathway different from that of the wt channel. Presently, the nature of dysfunctions (exaggerated or decreased expression and/or activity) affecting the KCNQ2wt, KCNQ2 splice variants and the PP2A (via its Bγ subunit) in BD onset or development is not clear.

Lithium is the most commonly used drug in the treatment of BD.[43] Figure 4a shows the different steps at which Li^+ can be expected to interfere with activity and regulation of the KCNQ2 channel. First, Li^+ inhibits several cellular enzymes involved in inositol recycling, a crucial step in the synthesis of the PIP2,[43] a membrane phospholipid that is essential for KCNQ2 activity and for its regulation by acetylcholine as well as by other neurotransmitters and peptides.[44–46] Li^+ by interfering with this pathway of regulation will necessarily interfere with KCNQ2 activity either basic or regulated by neurotransmitters such as acetylcholine acting on the M1 receptor. Second, the kinase GSK3β has also been shown to be inhibited by Li^+[43,47] and we have shown in this paper that GSK3β can, *in vitro*, phosphorylate KCNQ2 and that this phosphorylation is decreased by Li^+. Furthermore, we show that a lithium treatment works in synergy with the PP2A-Bγ subunit to increase the KCNQ2wt current, indicating that Li^+ also alters phosphorylation of the channel probably by inhibiting the endogenous GSK3β activity. Additionally, Li^+

has no effect on splice variant activity which could be related to the lack of efficacy of the lithium treatment in a significant number of patients.[43]

In summary, our results suggest that a dysfunction of the KCNQ2-PP2A-*Bγ* subunit regulation pathway and/or a dysfunction in the KCNQ2/KCNQ2 splice variant expression and association might be responsible for changes in neuronal excitability that underline the manic and the depressive episodes occurring in BD. It would be particularly important to determine whether individuals who displayed BFNC in their early childhood are more prone to BD as adults. Seizures in these individuals usually start around day 3 post-natal and disappear in 85–90% of the patients after a few months and only 10–15% of the patients display seizures later in childhood.[18] Both results presented in this work for KCNQ2 and recent data implicating KCNQ3[39] that can also be mutated in BFNC[38] plead for the interest of such a clinical investigation.

The most important conclusion of this work is that it points to the KCNQ2 channel, and its splice variants, as potentially interesting therapeutical targets. The pharmacology of this particular channel type has already started to be investigated as the channel might also be of interest in neuropathic pain,[48] epilepsy[49,50] and to improve memory.[42]

Materials and methods

Genetic analysis

The possible association of PP2A with bipolar disorder was analyzed in two different populations. One sample was provided by Hospital Pinero, Buenos-Aires, Argentina (the 'Labimo' collection). The other sample was provided by the University College of London (the 'UCL' collection). Both collections are composed of patients affected by bipolar disease according to DSM-IV criteria, and their matched healthy controls. Drug-abusing patients were excluded.

Two hundred and six DNA samples from unrelated patients suffering from bipolar disorder and 201 DNA control samples were collected from the Labimo collection, for genotyping analysis. Three hundred and fifteen and 300 DNA samples were analyzed from the UCL collection.

Genotyping of affected and control individuals was obtained by scanning the DNA sample from all individuals in order to establish the allele frequencies of biallelic markers. Thus, we determined the frequencies of every biallelic marker in each population (cases and controls) performing microsequencing reactions on amplified fragments obtained by genomic PCR performed on the DNA samples from each individual.

A detailed experimental procedure for genetic analysis is given in on-line Supplementary information.

Materials

Marathon–Ready human brain cDNA library and human brain thalamus polyA+ RNA were from BD Biosciences (Pont-de-Claix, France) Quick change site-directed mutagenesis kit was from Stratagene (Amsterdam, The Netherlands).

Y2H screening and yeast mating tests

The *Saccharomyces cerevisiae* AH109 strain was transformed with pGBKT7 plasmid (BD Biosciences) containing the 1644 bp cDNA (genbank accession number: AF086924) of the *PPP2R2C* gene according to the manufacturer's instructions (Matchmaker Two-Hybrid system, BD Biosciences). Cells expressing the bait were then mixed with a pretransformed Matchmaker human brain cDNA library in the Y187 strain. Three independent matings were performed with, respectively, 5×10^6, 5×10^6 and 2×10^5 clones of the human brain cDNA library. The resulting diploid cells able to grow on SD/-Leu/-Trp medium containing plates were further selected onto the medium-stringency SD/-Leu/-Trp/-His-selective medium for the identification of bait—prey interactions. Positive colonies were then plated onto the high-stringency SD/-Leu/-Trp/-His/-Ade-selective medium. Only cDNA of colonies able to grow at the same time on both media were retained for further studies

In vitro *or* ex vivo *GST pull-down assay*

In vitro binding assays with GST-3H2, GST-KCNQ2wtC-ter and GST-PPP2R2C fusion proteins were performed essentially as described in Cavarec *et al.*[51] The pGBKT7-3H2 and pGBKT7-PPP2R2C plasmids were *in vitro* transcribed and translated (TNT T7 Quick coupled transcription/translation system, Promega, Charbonnieres-Les-Bains, France) in the presence of L-[^{35}S] methionine.

In vitro *phosphorylation assay with recombinant GSK3β kinase and dephosphorylation with U-87 MG whole cell extracts*

Beads with bound fusion proteins were washed three times in NETN buffer (120 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0 and 0.5% Nonidet P-40) and once in HMK buffer without dithiothreitol (DTT) (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 12 mM $MgCl_2$). Beads were resuspended in 240 μl of reaction mix (1 × HMK buffer with 20 mM DTT, 40 U of PKA catalytic subunit, 250 U/vial (Sigma, Saint-Quentin-Fallavier, France), in 40 mM DTT, 5 mM ATP) and incubated for 30 min at room temperature. Beads were then washed three times in NETN buffer and once in GSKβ-reaction buffer, resuspended in 50 μl of reaction mix (1 × GSK3β reaction buffer, 10 μCi of [^{32}P]$_\gamma$ATP, 50 U of recombinant GSK3β (New England Biolabs, Ozyme, Saint-Quentin-en-Yvelines, France) and incubated at room temperature for 30 min with 25 mM LiCl. In competition experiments 2.5 μg of CREB phosphopeptides (New England Biolabs) were added to the kination mix. After three washes in NETN buffer, phosphorylated proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and visualized by autoradiography.

In dephosphorylation assays the *in vitro* phosphorylated GST-3H2 fusion protein was incubated at room temperature for 30 min with 500 μg of whole cell extracts of the U-87 MG human glioblastoma cell line (ATCC number: HTB-14) with or without 400 nM of the PP2A phosphatase inhibitor okadaic acid (Sigma). Phosphorylated proteins were resolved on 10% SDS-PAGE, and visualized by autoradiography.

Western blot analysis

HEK293-H cells were grown in Dulbecco's-modified Eagle's medium medium (Invitrogen, Cergy-Pontoise, France) supplemented with 0.1 mM non-essential amino acids and 10% fetal bovine serum (Invitrogen), and transiently transfected with 20 μg of either pCMV-Myc-Q2-3H9, pCMV-Myc-Q2-3H2, pcDNA3.1(+)-Q2-3H9-V5 or pcDNA3.1(+)-Q2-3H2-V5 plasmids per 60 mm dish using the Invitrogen calcium phosphate transfection protocols. Cell lysis performed in the presence of phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF), and Western blot analyses were carried out as in Cavarec *et al.*[51] with, as primary antibodies, anti-c-myc 9E10 monoclonal antibodies (working dilution (wd) 1:1000, Roche, Meylan, France), anti-KCNQ2 (N-19) polyclonal antibodies (wd 1:1000, Santa Cruz Biotechnology, Tebu SA, Le Peray-en-Yvelines, France), Anti-V5 monoclonal antibodies (wd 1:1000, Invitrogen), or Anti-HA monoclonal antibodies (wd 1:5000, Sigma).

Immunofluorescence analysis

At 24 h before transfection, HEK293-H cells were seeded onto poly-L-lysine-coated chamber slides (Lab-Tek) at a density of 2.5×10^5 cells per chamber. Cells were transfected with 0.8 μg of the pcDNA3.1(+)-Q2-3H9-V5 or pcDNA3.1(+)-Q2-3H2-V5 plasmids using the Lipofectamine 2000 (Invitrogen). After 2 days, cells were fixed for 10 min at room temperature with 4% paraformaldehyde (PAF) in 1 × phosphate-buffered saline (PBS). Permeabilized cells were obtained with 0.2% Triton X-100 in PBS for 5 min at room temperature. Incubations with 10% normal goat serum and 1% bovine serum albumin Fraction V (Sigma) in PBS containing 0.2% Triton X-100 and 0.02% Na Azide at room temperature for 1 h, were used to block non-specific sites. An anti-V5 fluorescein isothiocyanate (FITC) antibody (wd 1/200, Invitrogen) was used to detect tagged channels. After washes, cells were mounted in Prolong Antifade Kit reagent (Molecular Probes, Invitrogen, Cergy-Pontoise, France) containing 4'-6-diamidino-2-phenylindole (DAPI) (Sigma) at 200 ng/ml and observed by direct phase microscopy (Leica DMRXA, Leica microsystems, Rueil-Malmaison, France). Images were captured with a dual mode cooled CCD camera (Hamamatsu, Massy, France).

Electrophysiological measurements

Cells were seeded in 35 mm diameter Petri dishes at a density of 20 000 cells/dish for COS-7 cells and of 40 000 cells/dish for CHO and HEK-293 cells, 24 h prior to transfection. COS-7 cells were transfected using the classical DEAE-dextran method, CHO cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and HEK-293 cells using the calcium phosphate transfection protocol. Typically, per 35 mm Petri dish, 1 μg of KCNQ2wt or 1.5 μg of splice channel and 2 μg of GSK3β constructs were co-transfected. Cells were tested 24–48 h after transfection using the whole-cell configuration of the patch-clamp technique. Experiments were carried out at room temperature (20–25°C) following the procedure described in Tinel *et al.*[31] The voltage protocol was constituted by step depolarizations of long duration (2 s) from −60 mV to +100 mV (increment 10 mV). The holding potential was maintained at −80 mV.

Acknowledgments

We are very grateful to Dr Thomas Jentsch for the generous gift of the KCNQ2wt channels and Dr Gary Buell for helpful discussion. We thank Martine Jodar, Nathalie Leroudier, Aurélie Kiers and Franck Aguila for their expert technical assistance, Linda Belo for reading the manuscript and Yvette Benhamou for secretarial assistance.

Duality of Interest

None declared.

References

1 Goodwin FK. From the alcohol, drug abuse, and mental health administration. *JAMA* 1990; **264**: 2495.
2 Blackwood DH, He L, Morris SW, McLean A, Whitton C, Thomson M *et al.* A locus for bipolar affective disorder on chromosome 4p. *Nat Genet* 1996; **12**: 427–430.
3 Detera-Wadleigh SD, Badner JA, Berrettini WH, Yoshikawa T, Goldin LR, Turner G *et al.* A high-density genome scan detects evidence for a bipolar-disorder susceptibility locus on 13q32 and other potential loci on 1q32 and 18p11.2. *Proc Natl Acad Sci USA* 1999; **96**: 5604–5609.
4 Polymeropoulos MH, Schaffer AA. Scanning the genome with 1772 microsatellite markers in search of a bipolar disorder susceptibility gene. *Mol Psychiatr* 1996; **1**: 404–407.
5 Ewald H, Degn B, Mors O, Kruse TA. Support for the possible locus on chromosome 4p16 for bipolar affective disorder. *Mol Psychiatr* 1998; **3**: 442–448.
6 Als TD, Dahl HA, Flint TJ, Wang AG, Vang M, Mors O *et al.* Possible evidence for a common risk locus for bipolar affective disorder and schizophrenia on chromosome 4p16 in patients from the Faroe Islands. *Mol Psychiatr* 2004; **9**: 93–98.
7 Swift RG, Polymeropoulos MH, Torres R, Swift M. Predisposition of Wolfram syndrome heterozygotes to psychiatric illness. *Mol Psychiatr* 1998; **3**: 86–91.
8 Kato T, Iwamoto K, Washizuka S, Mori K, Tajima O, Akiyama T *et al.* No association of mutations and mRNA expression of WFS1/wolframin with bipolar disorder in humans. *Neurosci Lett* 2003; **338**: 21–24.
9 Abou Jamra R, Schumacher J, Golla A, Richter C, Otte AC, Schulze TG *et al.* Family-based association studies of alpha-adrenergic receptor genes in chromosomal regions with linkage to bipolar affective disorder. *Am J Med Genet B Neuropsychiatr Genet* 2004; **126**: 79–81.
10 Kirov G, Jones I, McCandless F, Craddock N, Owen MJ. Family-based association studies of bipolar disorder with candidate genes involved in dopamine neurotransmission: DBH, DAT1, COMT, DRD2, DRD3 and DRD5. *Mol Psychiatr* 1999; **4**: 558–565.
11 Muir WJ, Thomson ML, McKeon P, Mynett-Johnson L, Whitton C, Evans KL *et al.* Markers close to the dopamine D5 receptor gene (DRD5) show significant association with schizophrenia but not bipolar disorder. *Am J Med Genet* 2001; **105**: 152–158.
12 Strack S, Zaucha JA, Ebner FF, Colbran RJ, Wadzinski BE. Brain protein phosphatase 2A: developmental regulation and distinct cellular and subcellular localization by B subunits. *J Comp Neurol* 1998; **392**: 515–527.
13 Trojanowski JQ, Lee VM. Phosphorylation of paired helical filament tau in Alzheimer's disease neurofibrillary lesions: focusing on phosphatases. *FASEB J* 1995; **9**: 1570–1576.
14 Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, Caron MG. An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* 2005; **122**: 261–273.
15 Chumakov I, Blumenfeld M, Guerassimenko O, Cavarec L, Palicio M, Abderrahim H *et al.* Genetic and physiological data implicating the new

human gene G72 and the gene for D-amino acid oxidase in schizophrenia. *Proc Natl Acad Sci USA* 2002; **99**: 13675–13680.
16 Hoshi N, Zhang JS, Omaki M, Takeuchi T, Yokoyama S, Wanaverbecq N *et al.* AKAP150 signaling complex promotes suppression of the M-current by muscarinic agonists. *Nat Neurosci* 2003; **6**: 564–571.
17 Gargus JJ. Unraveling monogenic channelopathies and their implications for complex polygenic disease. *Am J Hum Genet* 2003; **72**: 785–803.
18 Biervert C, Schroeder BC, Kubisch C, Berkovic SF, Propping P, Jentsch TJ *et al.* A potassium channel mutation in neonatal human epilepsy. *Science* 1998; **279**: 403–406.
19 Singh NA, Charlier C, Stauffer D, DuPont BR, Leach RJ, Melis R *et al.* A novel potassium channel gene, KCNQ2, is mutated in an inherited epilepsy of newborns. *Nat Genet* 1998; **18**: 25–29.
20 Biervert C, Steinlein OK. Structural and mutational analysis of KCNQ2, the major gene locus for benign familial neonatal convulsions. *Hum Genet* 1999; **104**: 234–240.
21 Maljevic S, Lerche C, Seebohm G, Alekov AK, Busch AE, Lerche H. C-terminal interaction of KCNQ2 and KCNQ3 K+ channels. *J Physiol* 2003; **548**: 353–360.
22 Schwake M, Jentsch TJ, Friedrich T. A carboxy-terminal domain determines the subunit specificity of KCNQ K+ channel assembly. *EMBO Rep* 2003; **4**: 76–81.
23 Nakajo K, Kubo Y. Protein kinase C shifts the voltage dependence of KCNQ/M channels expressed in Xenopus oocytes. *J Physiol* 2005; **569**: 59–74.
24 Bhat RV, Budd Haeberlein SL, Avila J. Glycogen synthase kinase 3: a drug target for CNS therapies. *J Neurochem* 2004; **89**: 1313–1317.
25 O'Brien WT, Harper AD, Jove F, Woodgett JR, Maretto S, Piccolo S *et al.* Glycogen synthase kinase-3beta haploinsufficiency mimics the behavioral and molecular effects of lithium. *J Neurosci* 2004; **24**: 6791–6798.
26 Kennelly PJ, Krebs EG. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J Biol Chem* 1991; **266**: 15555–15558.
27 Schroeder BC, Kubisch C, Stein V, Jentsch TJ. Moderate loss of function of cyclic-AMP-modulated KCNQ2/KCNQ3 K+ channels causes epilepsy. *Nature* 1998; **396**: 687–690.
28 Visscher PM, Haley CS, Ewald H, Mors O, Egeland J, Thiel B *et al.* Joint multi-population analysis for genetic linkage of bipolar disorder or 'wellness' to chromosome 4p. *Am J Med Genet B Neuropsychiatr Genet* 2005; **133**: 18–24.
29 Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* 2001; **353**: 417–439.
30 Meyre D, Bouatia-Naji N, Tounian A, Samson C, Lecoeur C, Vatin V *et al.* Variants of ENPP1 are associated with childhood and adult obesity and increase the risk of glucose intolerance and type 2 diabetes. *Nat Genet* 2005; **37**: 863–867.
31 Tinel N, Lauritzen I, Chouabe C, Lazdunski M, Borsotto M. The KCNQ2 potassium channel: splice variants, functional and developmental expression. Brain localization and comparison with KCNQ3. *FEBS Lett* 1998; **438**: 171–176.
32 Tinel N, Diochot S, Lauritzen I, Barhanin J, Lazdunski M, Borsotto M. M-type KCNQ2-KCNQ3 potassium channels are modulated by the KCNE2 subunit. *FEBS Lett* 2000; **480**: 137–141.
33 Schmidt K, Kins S, Schild A, Nitsch RM, Hemmings BA, Gotz J. Diversity, developmental regulation and distribution of murine PR55/B subunits of protein phosphatase 2A. *Eur J Neurosci* 2002; **16**: 2039–2048.
34 Weber YG, Geiger J, Kampchen K, Landwehrmeyer B, Sommer C, Lerche H. Immunohistochemical analysis of KCNQ2 potassium channels in adult and developing mouse brain. *Brain Res* 2006; **1077**: 1–6.
35 Chouabe C, Neyroud N, Guicheney P, Lazdunski M, Romey G, Barhanin J. Properties of KvLQT1 K+ channel mutations in Romano–Ward and Jervell and Lange–Nielsen inherited cardiac arrhythmias. *EMBO J* 1997; **16**: 5472–5479.
36 Wang Q, Curran ME, Splawski I, Burn TC, Millholland JM, VanRaay TJ *et al.* Positional cloning of a novel potassium channel gene: KVLQT1 mutations cause cardiac arrhythmias. *Nat Genet* 1996; **12**: 17–23.
37 Jentsch TJ, Schroeder BC, Kubisch C, Friedrich T, Stein V. Pathophysiology of KCNQ channels: neonatal epilepsy and progressive deafness. *Epilepsia* 2000; **41**: 1068–1069.
38 Jentsch TJ. Neuronal KCNQ potassium channels: physiology and role in disease. *Nat Rev Neurosci* 2000; **1**: 21–30.
39 Avramopoulos D, Willour VL, Zandi PP, Huo Y, MacKinnon DF, Potash JB *et al.* Linkage of bipolar affective disorder on chromosome 8q24: follow-up and parametric analysis. *Mol Psychiatr* 2004; **9**: 191–196.
40 Pan Z, Selyanko AA, Hadley JK, Brown DA, Dixon JE, McKinnon D. Alternative splicing of KCNQ2 potassium channel transcripts contributes to the functional diversity of M-currents. *J Physiol* 2001; **531**: 347–358.
41 Xie X, Hagan RM. Cellular and molecular actions of lamotrigine: possible mechanisms of efficacy in bipolar disorder. *Neuropsychobiology* 1998; **38**: 119–130.
42 Peters HC, Hu H, Pongs O, Storm JF, Isbrandt D. Conditional transgenic suppression of M channels in mouse brain reveals functions in neuronal excitability, resonance and behavior. *Nat Neurosci* 2005; **8**: 51–60.
43 Quiroz JA, Gould TD, Manji HK. Molecular effects of lithium. *Mol Interv* 2004; **4**: 259–272.
44 Suh BC, Hille B. Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. *Neuron* 2002; **35**: 507–520.
45 Zhang H, Craciun LC, Mirshahi T, Rohacs T, Lopes CM, Jin T *et al.* PIP(2) activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. *Neuron* 2003; **37**: 963–975.
46 Delmas P, Brown DA. Pathways modulating neural KCNQ/M (Kv7) potassium channels. *Nat Rev Neurosci* 2005; **6**: 850–862.
47 Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci USA* 1996; **93**: 8455–8459.
48 Cooper EC, Jan LY. M-channels: neurological diseases, neuromodulation, and drug development. *Arch Neurol* 2003; **60**: 496–500.
49 Peretz A, Degani N, Nachman R, Uziyel Y, Gibor G, Shabat D *et al.* Meclofenamic acid and diclofenac, novel templates of KCNQ2/Q3 potassium channel openers, depress cortical neuron activity and exhibit anticonvulsant properties. *Mol Pharmacol* 2005; **67**: 1053–1066.
50 Passmore GM, Selyanko AA, Mistry M, Al-Qatari M, Marsh SJ, Matthews EA *et al.* KCNQ/M currents in sensory neurons: significance for pain therapy. *J Neurosci* 2003; **23**: 7227–7236.
51 Cavarec L, Kamphausen T, Dubourg B, Callebaut I, Lemeunier F, Metivier D *et al.* Identification and characterization of Moca-cyp. A *Drosophila melanogaster* nuclear cyclophilin. *J Biol Chem* 2002; **277**: 41171–41182.

Supplementary Information accompanies the paper on the The Pharmacogenomics Journal website (http://www.nature.com/tpj)